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Crystallization and preliminary X-ray crystallographic analysis of blood coagulation factor V-activating proteinase (RVV-V) from Russell's viper venom

Daisuke Nakayama, Youssef Ben Ammar and Soichi Takeda*

Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Japan

Correspondence e-mail: stakeda@ri.ncvc.go.jp

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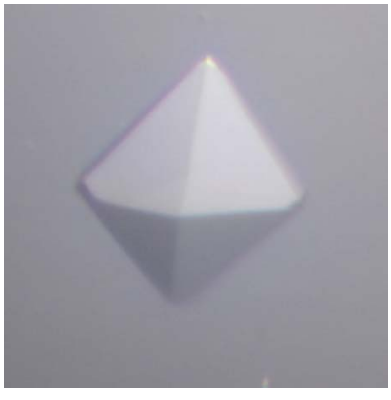
Russell's viper venom blood coagulation factor V activator (RVV-V) is a thrombin-like serine proteinase that specifically activates factor V by cleaving a single peptide bond between Arg1545 and Ser1546. Activated factor V combines with activated factor X produced by the enzyme RVV-X in the venom to form the prothombinase complex, which can induce disseminated intravascular coagulopathy in envenomated animals. In the current study, RVV-V was crystallized in order to attempt to understand its substrate specificity for factor V. Four distinct crystal forms of RVV-V were obtained using the sitting-drop vapour-diffusion method and diffraction data sets were collected on SPring-8 beamlines. The best crystal of RVV-V generated data sets to 1.9 Å resolution.

1. Introduction

Blood coagulation factor V is a key component of the haemostatic system (Nicolaes & Dahlback, 2002). Factor V exists as a precursor molecule in the circulation and is converted to its active form, factor Va, after cleavage of the three peptide bonds between Arg709 and Ser710, between Arg1018 and Thr1019 and between Arg1545 and Ser1546 by thrombin or activated factor X (factor Xa; Mann & Kalafatis, 2003; Thorelli *et al.*, 1998). Factor Va acts as a nonenzymatic cofactor in the prothrombinase complex that converts prothrombin to thrombin. Factor Va also enhances the rate of prothrombin activation of factor Xa on phospholipid membranes. This rate is enhanced by about 300 000-fold and enhancement occurs in a Ca^{2+}-dependent manner (Nesheim *et al.*, 1979; Rosing *et al.*, 1980). Finally, thrombin converts soluble fibrinogen into insoluble fibrin, leading to blood-clot formation.

The venom of Russell's viper (*Daboia russelli siamensis*) has been recognized for its potent coagulation activity. Two major components of Russell's viper venom can collaboratively accelerate disseminated intravascular coagulation in the body of prey (Schiffman *et al.*, 1969). One of these components is RVV-X (EC 3.4.24.58), a heterotrimeric metalloproteinase that specifically activates factor X. We recently determined the crystal structure of RVV-X at 2.9 Å resolution and proposed a model for factor-X activation (Takeda *et al.*, 2007). The other component is RVV-V (EC 3.4.21.95), a thrombin-like serine proteinase that specifically activates factor V (Hjort, 1957). RVV-V cleaves the single peptide bond between Arg1545 and Ser1546, resulting in activation of factor V (Dahlback, 1986; Kane & Davie, 1986, 1988; Jenny *et al.*, 1987). RVV-V does not cleave the other two thrombin-susceptible sites of factor V. Furthermore, RVV-V is resistant to endogenous serine proteinase inhibitors (serpins) such as antithrombin, which regulates thrombin activity under physiological conditions (Segers *et al.*, 2006).

The molecular mechanism by which RVV-V recognizes and cleaves the Arg1545–Ser1546 bond of factor V is poorly understood, primarily owing to a lack of high-resolution structural information, although a homology model of RVV-V has previously been reported (Segers *et al.*, 2006). To gain insight into the molecular basis of its substrate recognition and serpin resistance, we crystallographically analyzed RVV-V. Here, we report the crystallization and preliminary crystallographic studies of RVV-V with and without inhibitors.



2. Materials and methods

2.1. Purification

D. russelli siamensis venom was purchased from the Japan Snake Institute. RVV-V was purified as described previously (Schiffman *et al.*, 1969; Kisiel, 1979) with some modifications. Lyophilized crude venom powder (200 mg) was dissolved in TBS buffer (10 m*M* Tris–HCl pH 7.5 and 150 m*M* NaCl) to a final concentration of 40 mg ml^{-1} and centrifuged at 15 000*g* for 30 min. The supernatant was loaded onto a HiPrep 26/60 Sephacryl S-100 HR column (GE Healthcare, UK) equilibrated with TBS buffer. Fractions containing RVV-V were pooled and applied onto a 1 ml Resource S column (GE Healthcare) pre-equilibrated with TBS buffer. Nonspecifically bound proteins were eluted with washing buffer (10 m*M* Tris–HCl pH 7.5 and 200 m*M* NaCl pH 7.5) and bound proteins were eluted with a linear gradient of NaCl (200–600 m*M*) in 10 m*M* Tris–HCl pH 7.5. The fractions containing RVV-V were pooled, dialyzed against 10 m*M* Tris–HCl, concentrated using Vivaspin (Sartorius, Germany) with a 10 000 nominal molecular-weight-limit membrane to give a protein concentration of 11.3 mg ml^{-1} and subjected to crystallization.

2.2. Crystallization

Preliminary screening of the crystallization conditions was performed with PEG/Ion 1, PEG/Ion 2, Crystal Screen 1 and Crystal Screen 2 kits (Hampton Research, USA) using the sitting-drop vapour-diffusion method at 277 and 293 K. Protein aliquots (0.1 µl at a concentration of 11.3 mg ml^{-1}) with or without the inhibitors Pefabloc [4-(2-aminoethyl)-benzenesulfonyl fluoride; 10 m*M*; Roche, Switzerland] or PPACK (D-Phe-Pro-Arg-chloromethylketone; 10 m*M*; Calbiochem, Germany) were mixed with 0.1 µl reservoir solution using a repeatable nanolitre pipetting device (Mosquito, TTP Labotech, UK). Droplets were equilibrated against 100 µl reservoir solution.

2.3. Crystallographic data collection

For X-ray measurements, crystals were cryoprotected, mounted in a nylon loop (Hampton Research, USA) or a Lytho Loop (ProteinWave Corp., Japan) and immediately exposed to a stream of nitrogen gas at 100 K to flash-freeze the samples. The crystals were evaluated in-house using Cu $K\alpha$ radiation (λ = 1.5418 Å) generated by an RA-Micro 7 rotating-anode X-ray generator with an R-AXIS VII image-plate detector (Rigaku, Japan). High-resolution data sets were collected using a Rayonix MX225HE CCD detector installed on the BL41XU beamline at SPring-8. The images collected were processed using the *HKL*-2000 software (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Purification

RVV-V was purified from the crude venom of *D. russelli siamensis* using gel-filtration chromatography followed by cation-exchange chromatography. The homogeneity of the purified RVV-V was confirmed by SDS–PAGE in the presence or absence of 10 m*M* dithiothreitol (Laemmli, 1970). RVV-V consists of 236 amino acids with a molecular weight of 29 kDa (Tokunaga *et al.*, 1988). Consistent with this previous report, after two-step chromatography the purified RVV-V band was detected at about 29 kDa (Fig. 1). Approximately 4 mg RVV-V was purified from 200 mg crude venom.

3.2. Crystallization

RVV-V crystals were initially obtained under two distinct conditions. Form 1 crystals were obtained using solution No. 38 of the PEG/Ion 2 kit [50 m*M* citric acid, 50 m*M* bis-tris propane pH 5.0 and 20%(*w*/*v*) PEG 3350] at 293 K. Form 2 crystals were obtained using solution No. 47 of the PEG/Ion 2 kit [1%(*w*/*v*) tryptone, 0.05 *M* Na HEPES pH 7.0 and 12%(*w*/*v*) PEG 3350] at 293 K. Crystals of

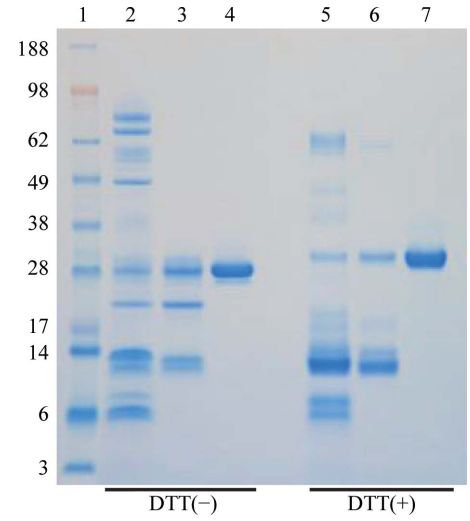


Figure 1
Purification of RVV-V. Crude venom (20 µg) and purified RVV-V (4 µg) were loaded on 4–12% SDS–PAGE in the presence or absence of 10 m*M* dithiothreitol. Proteins were stained with Coomassie Brilliant Blue. Lane 1, molecular-weight markers (kDa). Lanes 2 and 5, crude venom from *D. russelli siamensis*. Lanes 3 and 6, RVV-V purified by gel-filtration chromatography. Lanes 4 and 7, RVV-V purified by cation-exchange chromatography. Lanes 2–4 are under nonreducing conditions [DTT(−)] and lanes 5–7 are under reducing conditions [DTT(+)].

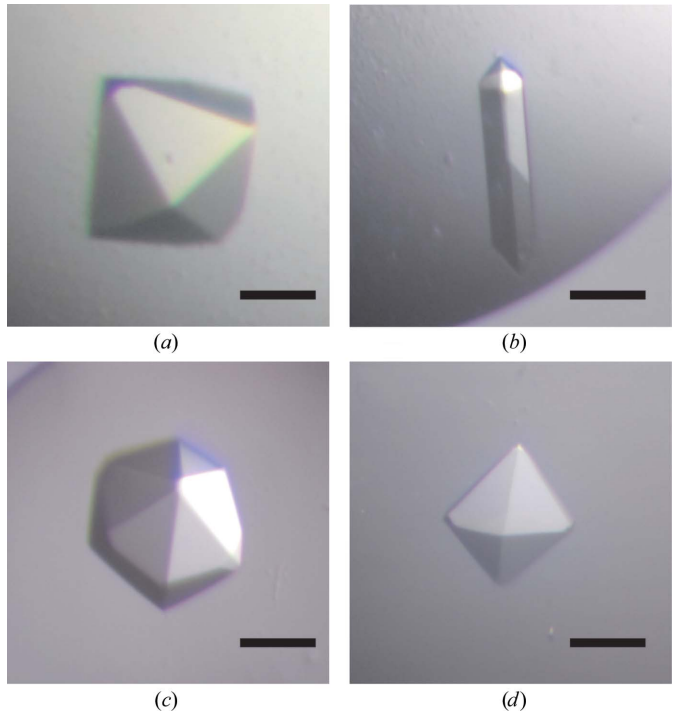


Figure 2
Crystals of RVV-V obtained under different conditions. (*a*) Form 1, (*b*) form 2, (*c*) form 3 (complexed with Pefabloc) and (*d*) form 4 (complexed with PPACK). The scale bar is 0.1 mm in length.

Table 1
Data-collection statistics for RVV-V crystals.

Values in parentheses are for the highest resolution shell.

	Form 1	Form 2	Form 3	Form 4
Inhibitor	None	None	Pefabloc	PPACK
X-ray source	SPring-8 BL41XU			
Space group	$P6_522$	$P6_522$	$P6_522$	$P6_522$
Unit-cell parameters				
a (Å)	78.9	80.1	78.9	77.2
b (Å)	78.9	80.1	78.9	77.2
c (Å)	157.3	160.4	160.6	168.4
$\alpha = \beta$ (°)	90	90	90	90
γ (°)	120	120	120	120
Resolution (Å)	50–1.9 (1.97–1.9)	50–1.9 (1.97–1.9)	30–2.85 (2.95–2.85)	30–2.55 (2.64–2.55)
No. of reflections	23547 (2301)	24182 (2389)	7400 (708)	10311 (993)
$R_{\rm merge}$†	0.06 (0.255)	0.05 (0.282)	0.06 (0.319)	0.06 (0.267)
$I/\sigma(I)$	22.8 (8.64)	22.0 (7.13)	40.6 (12.3)	33.8 (15.8)
Completeness (%)	99.8 (100)	97.6 (99.5)	99.9 (100)	99.7 (100)
Redundancy	7	7.2	20.7	20.4
No. of molecules in ASU	1	1	1	1
Matthews value (Å^3 Da^{-1})	2.66	2.78	2.70	2.69
Solvent content (%)	53.9	55.8	54.6	54.2

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl and $\langle I(hkl)\rangle$ is its average.

the RVV-V–Pefabloc and RVV-V–PPACK complexes were initially obtained in a similar manner as form 1 crystals using solution No. 38 of the PEG/Ion 2 kit at 293 K.

Because the crystals obtained from the initial screen were too small for X-ray analysis, we optimized the crystallization conditions to obtain larger single crystals by lowering the precipitant concentration and changing the droplet size and pH. Larger form 1 crystals were obtained by mixing 0.5 µl protein solution and 0.5 µl of a reservoir solution containing 50 m*M* citric acid, 50 m*M* bis-tris propane pH 5.0 and 16%(*w*/*v*) PEG 3350 (Fig. 2*a*). Larger form 2 crystals were obtained by mixing 0.3 µl protein solution and 0.3 µl of a reservoir solution containing 0.8%(*w*/*v*) tryptone, 0.04 *M* Na HEPES pH 7.0 and 9.6%(*w*/*v*) PEG 3350 (Fig. 2*b*). Diffraction-quality crystals of form 3 (RVV-V–Pefabloc complex) and form 4 (RVV-V–PPACK complex) were obtained by mixing 0.5 µl protein solution and 0.5 µl of a reservoir solution containing 0.8%(*w*/*v*) tryptone, 0.04 *M* Na HEPES pH 7.0 and 9.6%(*w*/*v*) PEG 3350 (Figs. 2*c* and 2*d*).

Crystals with dimensions of 200 × 200 × 200 µm (forms 1, 3 and 4) or 300 × 50 × 50 µm (form 2) were formed after 3–7 d at 293 K.

3.3. X-ray analysis

Prior to data collection, single crystals were cryoprotected using a reservoir solution supplemented with increasing glycerol concentrations (5, 10, 15 and 20%) in order to avoid osmotic shock-induced cracking. All diffraction data sets were acquired with a Rayonix MX225HE CCD detector using the oscillation method on beamline BL41XU, with an oscillation angle of 1.0°, a wavelength of 1.0 Å and a crystal-to-detector distance of 160 mm. The unit-cell parameters and statistics for the data sets are summarized in Table 1.

The asymmetric unit of each crystal was estimated to contain one RVV-V molecule, with corresponding crystal volume per protein weight ratios of 2.66, 2.78, 2.70 and 2.69 Å^3 Da^{-1} for crystal forms 1, 2, 3 and 4, respectively (Matthews, 1968). Solvent-content estimations based on a single copy of the molecule per asymmetric unit gave values of 53.9, 55.8, 54.6 and 54.2% for crystals forms 1, 2, 3 and 4, respectively. The unit-cell parameters of these four crystal forms were similar to, but distinct from, each other, suggesting structural changes that depended on the pH or on inhibitor binding. Clear molecular-replacement (MR) solutions for each crystal form were obtained in space group $P6_522$ using *MOLREP* from the *CCP*4 suite (Vagin & Teplyakov, 1997) with Ancrod (61% sequence identity), a snake-venom protein C activator from *Agkistrodon contortrix contortrix* (PDB code 2aiq; Murakami & Arni, 2005), as the structural model. Structural analyses of these crystals together with MR phasing are ongoing.

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